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THE RELATION OF *HERPETOMONAS ELMASSIANI* (MIGONE) TO ITS PLANT AND INSECT HOSTS.

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Herpetomonas elmassiani (Migone) is at present the only species of latex-inhabiting herpetomonad known in the United States. Its plant host is the common milkweed, *Asclepias syriaca* L., in the latex of which it has been found in great numbers in Maryland and New Jersey (Holmes, 1924, 1925*a*). Its suspected insect host in these locations is a red and black hemipterous insect, *Oncopeltus fasciatus* (Dall.). The same species of plant flagellate appears to inhabit other milkweeds in countries along the Atlantic coast of Central and South America. It has been reported from Haiti, Honduras and Paraguay, and doubtless exists at intermediate points between these countries and the locations in the United States. In southern locations other species of *Oncopeltus* frequenting the infected plants have seemed to act as insect hosts.

In view of the lack of apparent pathogenicity of the herpetomonads in Maryland milkweeds (Holmes, 1925*b*), it seems desirable to report upon the relation of the flagellate to the tissues of its hosts. The well known species, *Herpetomonas davidi* (Lafont), which inhabits the latex cells of Euphorbias in Europe and elsewhere, is pathogenic to its host (França, 1914, Nieschulz, 1922), in which it causes modifications of the latex cells and neighboring portions of the plant sufficient to stunt or kill whole branches or even whole plants. The reasons for the lack of harm resulting in the milkweed host from the presence of *Herpetomonas elmassiani* (Migone) may be the freedom from infection of some of the latex systems even of heavily infected

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plants, the entire freedom from penetration of tissues aside from the latex systems, and the sufficient food supply presented to the flagellates by the milky vacuole fluid itself in the latex cells.

The fact is often overlooked that plant flagellates of the type of *Herpetomonas davidi* (Lafont) and *Herpetomonas elmassiani* (Migone) have been found *only* in such plants as have abundant latex. Those engaged in research in this field are of course acquainted with this restriction of the range of the organisms, and take it into account in most of their work, but do not always seem to have it very definitely in mind. Others whose interest in plant flagellates arises from some other, less immediate, source are often entirely unmindful of the situation until it is brought to their attention.

To the present time no acceptable species of flagellates of the genera *Herpetomonas*, *Leishmania*, *Crithidia* or *Trypanosoma* have been found in plants other than those provided with a milky juice or latex.

Perhaps the situation would be better understood if it were commonly known that latex does not occur extracellularly in plants, but intracellularly. Thus the flagellates which are transferred by their insect hosts to the latex cells of plants are not to be found thereafter at random in the plant tissues, but are strictly intracellular (not intracytoplasmic) parasites.

A description of the cells containing the latex will make clear the relation of any latex-inhabiting organisms to the host plants.

The latex ducts of the plants with which protozoölogical studies have been most concerned are those known as simple ducts, because they do not fuse with each other in the course of their wanderings. Among the Asclepiadaceæ, Euphorbiaceæ, Apocynaceæ and Urticaceæ the cells destined to become the latex ducts of the mature plants are already distinguishable in the embryo. Their nuclei divide again and again, and the cells elongate tremendously and branch repeatedly, but no cross walls are formed, nor do the ducts fuse with one another, so that eventually the few original cells penetrate every part of the plant, and still remain distinct and separate from each other. The thin cellulose walls are lined with a layer of cytoplasm containing numerous nuclei. In the vacuole is collected the milky, usually white latex.

It is well to bear in mind that there is another type of latex system among plants, in which the original cells fuse together by the destruction of their partitions and cross-walls. The result

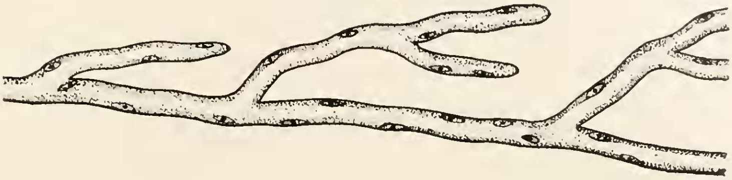

FIG. 1. Diagrammatic representation of a portion of a simple latex duct such as is found in the species of the genus *Asclepias*. The latex is secreted by the wall of cytoplasm into the extensive central vacuole. Such a duct will not fuse with others in the course of its growth.

of the process is the formation of interlacing vessels, in which the latex is contained. This type of latex system is found in such plants as lettuce.

Diagrams representing these two types of latex ducts are shown in Figs. 1 and 2.

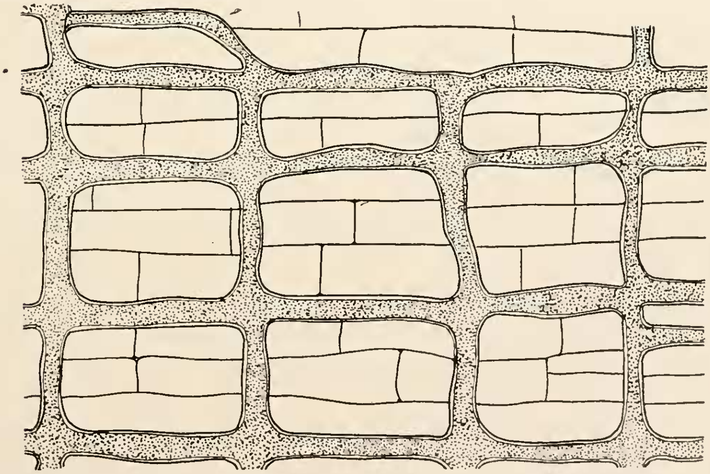

FIG. 2. Diagrammatic representation of a system of latex vessels such as is found in lettuce. The original cells fuse together to form a network, instead of remaining as independent latex ducts. The type of latex cell involved in protozoölogical studies has been that shown in Fig. 1.

The flagellates known to inhabit the latex systems of plants are probably all insect parasites. They enter the cells of the plant hosts only when the insects introduce their beaks directly into the latex to feed. In the cells they do not enter the cytoplasm, so far as can be seen, but inhabit the milky juice which fills the long cell vacuole. There they grow and multiply rapidly, as is indicated by the many dividing specimens present in latex smears from infected plants stained with Wright's stain. The flagellates are shut off from other cells of the plant and even from other latex cells of which there may be several. Unless a single plant were infected several times by insects it is unlikely that all the latex ducts would become infected, even though one or more might.

Localized Infections.

If one latex system of a plant is infected, a macroscopically localized infection may result unless that system happens to penetrate every leaf and flowerlet of the plant.

Early in the season of 1924 a search was made for such cases, for the previous year all the infections had appeared systemic.

It seemed likely that insects might repeatedly bite plants during the late summer, but that during June and early July any infections which might occur from insect carriage would be the result of a minimum number of infective bites.

The first example of the way in which this worked out in the field was met when a plant of a group surveyed thoroughly every few days gave a negative record after showing flagellates on several occasions. The later examinations of this plant showed that flagellates were present, but were not always to be found in the single drops of latex preserved as records. For this plant, then, a new system of sampling was instituted. Samples were taken from ten leaves instead of from one. It was found that some leaves were positive and others negative, just as one might reasonably expect if only a few of the latex cells were parasitized.

The study of sections of petiole and leaf tissue gathered at this time showed the even more interesting fact that in plants never suspected of having localized infections only a few of the latex cells were inhabited by organisms. It was easier to find negative cells than positive in specimens from apparently heavily parasitized plants. Smears from these plants showed very large numbers of organisms, yet when a droplet was taken for examination as much latex must have been contributed by uninfected cells as by infected. In the cells containing the organisms the concentration was so great that a moderate dilution was entirely ineffective in changing the appearance of the drop as stained for examination.

Since the plants above mentioned seemed when in the field to have systemic infections in that the slightest wound in any portion of the leaves or stems gave infected latex, and yet the microscopic examination of sections showed but a few of the cells infected, it is evident that the whole plants were penetrated by a relatively few single cells, and that if each individual latex cell fell short of extending completely throughout its plant, it still must have achieved very nearly this remarkable feat. This type of cell attains a notable total size and contains tremendous numbers of nuclei. Its latex-containing vacuole also is probably nearly if not quite the largest of cell vacuoles, making up in length far more than it lacks in breadth.

Confinement to Latex Cells.

Careful study failed to show any flagellates in the plant tissues outside the vacuoles of the latex cells. In some sections there were indeed abnormal and deceptive appearances caused by the flow of latex by capillary action along the conducting tissues. When pieces were cut to be fixed, latex always flowed out over the wounded surfaces. Since the turgidity of the plant was in part relieved by this loss, other specialized cells such as the spiral vessels were invaded by currents of latex along with which the flagellates themselves were carried. The abnormality of this process was made evident by examining the invaded ducts throughout their entire length. The flagellates were seen to be present only near the cut surfaces and to become less and less crowded as the distance from the exterior increased. The tissues in the interior presented a truer picture of conditions in the living plant.

Since the flagellates were normally confined to the latex cells, and indeed to the *latex* filling the long vacuoles, it became evident that certain observations made in the field were of more significance in their relation to the biology of the flagellate than had at first been realized.

It had been noticed that infections did not spread from plant to plant in groups connected by a common axis. Such plants arose from separate buds, the latex systems of which were independent of each other. In the axis itself no ducts occur, so that there is no chance for wandering through such a connection.

Late in the autumn of 1924 two plants which had been under observation for months were dug up to determine their exact relation to each other. One of these had been consistently negative for flagellates all summer. The other had been as consistently heavily infected in every part above the ground, with the exception of the seeds which are always free from invasion. The two plants were separated from each other by no more than six inches of axis, from which common source they had both sprung as buds. The absence of latex ducts in the axis and the confinement of the flagellates to the latex of the infected plant made it impossible that the nearby negative plant should be invaded except from some outside source of the organisms.

The practical confinement of the insect, *Oncopeltus fasciatus* (Dall.), suspected of being the insect host of the flagellate, to the blossoms and pods of the milkweed plant in feeding is also made significant by histological studies of the latex system, which in these two parts becomes much more prominent than it is in the stems or leaves. The soft tissues under the outer green coverings of the pods contain numerous branches of the latex system, and in the area between the pedicels and the bases of the petals of the flowers the latex ducts are exceedingly close together and voluminous. This offers a favorable feeding location to the insect, and, by reason of the crowded flagellates here in infected plants and the softness of the tissues, opportunity is offered for the infection of insects from the plant and for the infection of previously uninfected plants by the insects.

The Flagellates of *Oncopeltus fasciatus* (Dall.).

It is not yet known with certainty whether *Oncopeltus fasciatus* (Dall.) is the insect which carries the milkweed flagellate from one plant to another. But since there are several reasons for suspecting it to be the carrier, a study of its flagellates was undertaken. All along the coast of America, from Paraguay in the south to New Jersey in the north, wherever the flagellate has been found in the latex of the milkweeds some species of the genus *Oncopeltus* has been found feeding on the infected plants. These insects have always been more characteristic of the particular plants harboring the flagellates than any other type of insect. Just north of New Jersey, where no flagellates are known in the plants, another insect genus *replaces Oncopeltus*. The specimens of this which have been examined thus far have been entirely negative for any flagellate, though infected with Sporozoa both intracellularly and extracellularly in the salivary glands. This coincidence of the range of the milkweed flagellate with the range of *Oncopeltus fasciatus* (Dall.) suggests that the presence of the flagellate is dependent on the presence of the insect. Moreover *Oncopeltus* feeds characteristically on the pods and flowers of the plant, and since plants bearing seed are the ones to which the infection is practically limited it seems even more probable that *Oncopeltus* is the host. Another bit of circumstantial evidence is gained from the study of the morphology of the flagellates from the insect and from the plant. The plant flagellates are characterized by a twist in their ribbon-like bodies. This is rare in insect flagellates, but is found in the case of the parasites of *Oncopeltus*. The insect feeds on latex. The infected plants have swarms of flagellates in *their* latex in every portion of the upper parts of the plants. Thus the insect could easily become infected from his feeding. In the insect the histological studies about to be described have shown swarms of flagellates in definite lobes of the thoracic salivary gland. The secretion of this gland is led by a simple duct directly to the mouth parts during the process of feeding, so that there should be no great mechanical difficulty in the transfer of the insect's flagellates to the interior of the latex ducts of the plant.

It is natural then that in spite of the difficulties which have been experienced up to this time in attempting to obtain plant infections from the insects in regions where plant infections are not known in the field, efforts to establish the relation of the plant and insect flagellates by histological studies have been made. The results have been negative so far as obtaining definite proof of the identity of the two forms is concerned, but such interesting observations have been made during the study that they will be described here.

The first insects to be sectioned and stained were collected during 1923. At first the flagellates were overlooked, and that for two very good reasons. The principal search was made for them in the intestinal tract, where they do not occur in my material, if indeed they ever occur there. And the salivary gland forms, stained as carefully as they may be, never stand out with the clearness by which those in latex smears are characterized. For the process of drying and staining with Wright's stain, though open to the objection that the nuclear detail is lost, gives bright sharp pictures of the organisms, usually making them much darker than the background. This is far from the case with wet-fixed material, sectioned, and stained even with so good a stain as iron hæmatoxylin. By this process the nucleus may be stained with all the desired sharpness, but the background of salivary secretion retains the stain far more than does the flagellate's cytoplasm. So that usually one sees the body of the flagellate only as an unstained area surrounding the nucleus. The wall of the salivary gland also retains the stain so tenaciously that it must usually be left very black that the internal structure of the flagellate may be seen at its best. This makes the whole field very dark, with the object to be examined exceedingly delicate and lightly stained. When these first specimens of *Oncopeltus* were examined a second time for a different purpose, clusters of the flagellates were noted by chance on the wall of the gland. The slide under observation at the time was stained with a mixture of aniline dyes, but the finding was at once confirmed by iron hæmatoxylin slides of the same material.

During 1924 a quantity of material was obtained for sectioning to discover the extent of the infection among the insects during the late season when the plants were becoming more and more widely infected, to determine with certainty that the organ in question was really the salivary gland of the insect and not a salivary receptacle or some other organ, to see whether the organism present had more than one stage in its life-history in the insect, and to find exactly its relation to the salivary gland tissues and secretion.

The study of the morphology of the gland in which the flagellates occurred required complete series of serial sections of three insects. The gland of one of these was reconstructed section by section, for the lobes of the gland cannot be readily visualized from the separate sections. Sometimes but one or two show, at other times there appear to be five or six lobes because of the inclination of the section and the twists in the organ itself.

Histology of the Salivary Gland.

The salivary gland nearly fills the dorsal half of the thorax. Its efferent duct leads away from the point at which the three lobes come together, first running back toward the abdomen immediately under the gland, then turning and running forward. It dips beneath the œsophagus near its junction with the proventriculus, and beneath the ventral chain of nerve ganglia between the sub-œsophageal ganglion and the ganglion immediately posterior to it. From this point the duct runs forward to the mouthparts where its secretion enters the pump or syringe and the hypopharynx.

It was interesting to note that the three parts of the gland could be distinguished readily in iron hæmatoxylin eosin sections by the character of the secretion in each, in spite of the fact that the outline of each might be most deceptively placed according to the plane of the section. The anterior lobe contained frothy material staining pink with eosin. The ventral lobe was distended with a smooth or very slightly granular substance which also stained pink with eosin. It was thus easy to distinguish these two by the consistencies of their contents. The dorsal lobe was remarkable in that its fluid retained some of the iron hæmatoxylin stain and in addition picked up eosin eagerly, nearly attaining the brilliant orange red color characteristic of blood corpuscles in tissues stained for example with Delafield's (not Heidenhain's) hæmatoxylin and eosin.

Localization in Dorsal and Anterior Lobes.

It was in the third or dorsal lobe that the flagellates were really abundant. There they were often massed like a new tissue lining the entire lumen of the gland. The dark stain which the fluid took made it difficult to study the internal structure of the organisms. Yet they were so abundant that some were usually favorably located for observation. The flagellates lined the dorsal lobe most heavily near its posterior extremity. Nearer the efferent duct the organisms were often in groups or clusters, isolated a little from the rest of the mass, but closely bunched together with flagella attached to the wall.

In the anterior, foam-filled lobe there were a few flagellates always when they were present in the dorsal lobe. In the posterior lobe there were none, no matter how heavily the insect was parasitized.

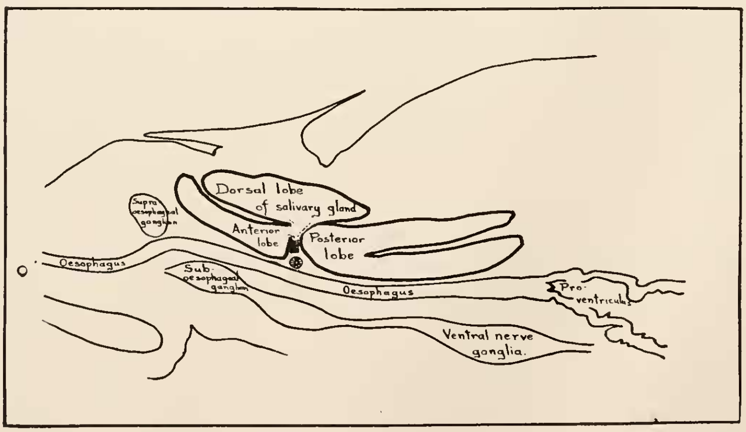

Fig. 3. Portion of a longitudinal section through *Oncopeltus*, showing the relative positions of the salivary gland and other thoracic organs. The three lobes of the gland come together in this particular section and the point of entrance of the salivary duct is shown.

That the fluids from the three lobes intermingled slightly at the common point of contact where the three fluids drained into the same efferent duct was indicated by the mixed consistencies and colors just at this exit point. In the smooth orange-red fluid a cloud of pink was always seen to have penetrated for a short distance. In the two pink portions, granular and frothy, red clouds were also visible just at the same point.

Until the gland had been reconstructed by drawing each section on blotting paper, cutting out and pasting together the individual sections, and coating the whole model with a beeswax and paraffin mixture, it was impossible to get an adequate idea of the complete gland. The lobes were so complicated in some planes of the section that it was not certain that no salivary reservoir entered the question. As the work was completed, however, it became evident that the structure in which the flagellates were located was a three-lobed thoracic salivary gland, with a single common exit. A diagram of the longitudinal section through the opening of the three lobes is to be seen in Fig. 3.

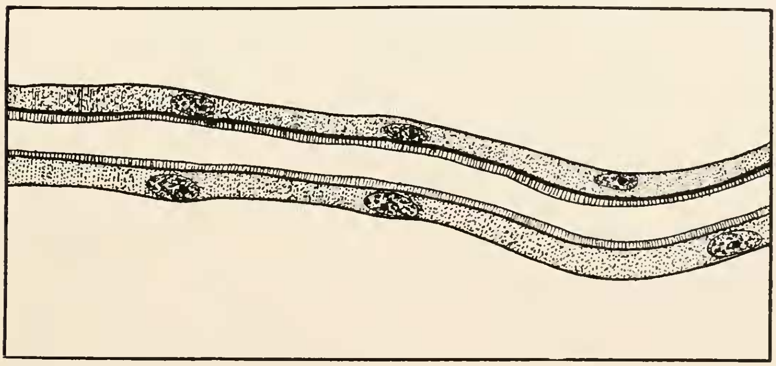

FIG. 4. A portion of the efferent duct of the salivary gland of *Oncopeltus*.

The cells of the duct are characteristic and interesting and with the secreting cells of the gland are so readily recognizable that no difficulty arises in identifying them even in sections which show but a tiny fragment of the salivary apparatus.

The duct is lined with a single layer of cells the nuclei of which are sometimes branched. A portion of the duct is represented in Fig. 4. A similar detailed drawing of the cells of the wall of the salivary gland is shown in Fig. 5. The glands are commonly distended with fluid. This escapes gradually, as required, through the efferent duct.

With the recognition of this organ as a thoracic salivary gland one of the objects of the histological examination of the insects was accomplished.

It was interesting to note that the flagellates always developed in the dorsal lobe most abundantly. A few penetrated the anterior, but none the ventral lobe. Evidently the differences in the compositions of the salivary secretions of the three lobes, indicated at once by the different staining reactions of the three portions, had also a significant effect on the organisms, inducing them to inhabit one portion more than others, and excluding them from the ventral lobe.

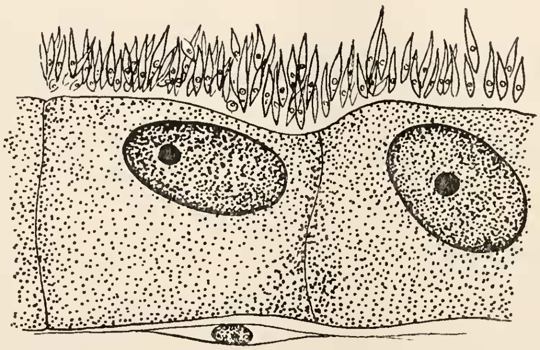

FIG. 5. A portion of the wall of the dorsal lobe of the salivary gland of an infected *Oncopeltus*. The nuclei of the gland cells are gigantic in comparison with the minute nuclei of the flagellates.

In my material no intestinal forms were found. This is remarkable when one considers that the type species of the genus *Herpetomonas* is a strictly intestinal flagellate in the common housefly. Salivary gland forms are the exception rather than the rule among Herpetomonads, and it would be expected that intestinal forms of the flagellate would be more noticeable than the gland forms. But if the flagellates of *Oncopeltus fasciatus* (Dall.) ever show intestinal forms I have not been able to secure the proper material to demonstrate them.

In addition to the complete series of sections of three individuals of *Oncopeltus fasciatus* (Dall.), a group of ten of the same species was sectioned and studied by choosing representative sections for staining and for examination. Sixteen individuals of *Lygæus kalmii* from Massachusetts were treated in the same way.

The object of using *Lygæus* was this: the limit of the range of *Oncopeltus fasciatus* coincides with the limit of the range of the plant flagellate, *Herpetomonas elmassiani* (Migone). But *Lygæus* is a very closely related insect, replacing *Oncopeltus* in the north where the flagellates are not found. It was desired to know whether it also had flagellates in its salivary glands. The thirteen specimens of *Oncopeltus*, which came from Maryland in September, 1924, at a time when the spread of plant flagellates was going on rapidly, were all infected with the exception of three individuals, which were rather young nymphs. The sixteen specimens of *Lygæus* were all negative for flagellates, both in the intestinal tract and in the salivary gland.

In the *Lygæus* examined there *were* infections with sporozoa in the glands, curiously enough in the same lobes frequented by the flagellates of *Oncopeltus*. One of the species present in *Lygæus* was occasionally seen also in *Oncopeltus* along with the flagellates. But in *Lygæus* the infections were much heavier, and decidedly destructive to the gland cells which were penetrated by intracellular stages and often rendered useless for secretion by the growth of the parasites and the consequent death of the cells.

The absence of flagellates in sixteen specimens of *Lygæus* collected at the end of the season was strikingly in contrast with the presence of large numbers of flagellates in the glands of ten of the thirteen *Oncopeltus* sectioned. If *Oncopeltus* is responsible for the spread of the milkweed flagellate it is no wonder that the spread is very rapid in September and early October when so large a percentage of the then rapidly multiplying insects are positive.

Of the original problems for the solution of which the histological work on *Oncopeltus* was carried out, but one remains for consideration. Does the flagellate of *Oncopeltus* have more than one stage of its life history in the insect? It seemed likely at first that there would be developmental stages of the herpetomonad in the intestine. But no colonization of the intestinal tract was indicated by any of my material. All the organisms were in the glands. The question then arose as to the exact position of the parabasal body in the forms colonizing the walls of the salivary gland. Careful study showed that in all cases in which the relative positions of nucleus and parabasal body could be definitely determined, the organism was constantly a herpetomonad. Smears of the insect flagellate confirmed this, for in them the position of the organelles could be determined in every single case far more readily than in sections of the gland. This question is not quite safely settled, for at some other season of the year the evidence might differ from that which I have been able to gather, but with the knowledge of the exact part of the insect parasitized, the dorsal and anterior thirds of the thoracic salivary gland, and the extent of infection during the season at which the insects are busily feeding on the infected plants, it will be easier to work on the questions concerned with the insect host of the flagellate.

Summary.

Histological studies of the milkweed host of the flagellate *Herpetomonas elmassiani* (Migone) showed that the organisms were confined to the latex system, in which they were intracellular but not intracytoplasmic. The latex is secreted into the general cell vacuole of the latex duct, and it is in this that the organisms were found. No other cells or parts of cells were found to be penetrated.

During the early part of the summer one or a very few latex cells in a plant were sometimes infected, for in *Asclepias* the original latex cells of the embryo never fuse. Because of this condition occasional localized infections appeared, in which a few leaves of the infected plant were found to be free from organisms.

The flagellates of *Oncopeltus fasciatus* (Dall.), a red and black hemipterous insect suspected of being the insect host of *H. elmassiani* (Migone), were found to inhabit the three-lobed thoracic salivary gland. In the gland these were definitely localized, colonizing only the dorsal and anterior lobes.

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